AD_____

GRANT NUMBER DAMD17-94-J-4270

TITLE: A New Generic Method for the Production of Protein-Based Inhibitors of Proteins Involved in Cancer Metastasis

PRINCIPAL INVESTIGATOR: Marshall H. Edgell, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina
At Chapel Hill
Chapel Hill, North Carolina 27599-4100

REPORT DATE: August 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Annual (1 Aug 97 - 31 Jul 98)

4. TITLE AND SUBTITLE
A New Generic Method for the Production of Protein-Based Inhibitors of Proteins Involved in Cancer Metastasis

5. FUNDING NUMBERS
DAMD17-94-J-4270

6. AUTHOR(S)
Marshall H. Edgell, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-4100

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The objective is to develop a method to make protein-based inhibitors against protein targets and as the test case to a proteinase involved in metastasis, stromelysin. The approach uses phage display and a display framework which should bind preferentially to the active site pocket of target proteins. We have now turned to construction technology which can be applied to any portion of the framework independent of restriction site distribution. A modified vector has been constructed and sequenced to support this approach. We have shown that peptides that bind to active stromelysin also bind to cadmium inactivated stromelysin which will not cleave weak binders allowing us to detect them and work to improve their affinity. In an effort to charaterize the binding epitope display framework, eglin c, we have also developed a new method for using mutagenesis to study protein structure which we call patterned library analysis. We have worked out a number of the technical details of employing this approach. In the process we have also discovered that eglin is reversibly denatured in low levels of SDS. This opens up the possibility of collecting thermodynamic parameters with an activity based assay that uses 100-fold less material than traditional biophysical techniques.

14. SUBJECT TERMS
Breast Cancer
stromelysin, phage display

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

MHE In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

MHE In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

MHE In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

MH Edgell 7-29-98

PI - Signature Date

Table of Contents

INTRODUCTION

Our objective is to learn how to efficiently build proteins, which will act as inhibitors to proteins involved in cancer metastasis. It is our expectation that such proteins can be constructed so as to be very specific for the desired target. While such inhibitors might be useful themselves as therapeutic molecules, they will certainly be useful as probes to define the issues associated with inactivating the target proteins; both the primary effects and side-effects.

The approach to be used in this project is to combine molecular genetics and protein biophysics to redirect to the target of interest the activity of a pre-existing protein, which will serve as a framework onto which to mount the desired modifications. Molecular genetics will be used to extend the reach of traditional protein engineering. The idea is to make large libraries of structural variants and then use genetic screening and selection strategies to find the best performers. Traditional protein biophysics will then be used to explore the various classes of variants and to make models for what is leading to inhibition. This information will then be used in subsequent cycles of design, construction and screening. We will also employ a cycle of mutation in a mutator strain of bacteria to evolve the inhibitor's affinity and specificity.

The development cycle that we will employ to modify the wild-type eglin c into a new inhibitor is:

1. make our best design guess as to what changes will increase binding to the desired target
2. construct a 'halo' of variants (~10^7) around the design
3. screen the variant library using phage display to find the best binders to the desired target
4. if affinities or specificity are not sufficient pursue two parallel paths to improvement
    - A. Biophysical analysis and redesign
        - i. characterize the binding classes using biophysical techniques (NMR, CD, ANS binding, etc.)
        - ii. use the biophysical information and modeling to build hypotheses concerning binding
        - iii. goto step 2 to carry out another cycle of screening
    - B. Laboratory Evolution
        - i. mutate the population of best binder in bacterial mutator strains
        - ii. goto step 3 to rescreen the population

The simplest inhibitors bind to their targets close to or at the active site and interfere with activity simply by getting in the way and not 'letting go' of the target. That is, such inhibitors lower the energy of the Michaelis complex sufficiently that very few molecules reach the transition state. These inhibitors can be designed to poke a projection into a groove in the target or enfold a pocket over a projection on the target. As our initial protein for protein engineering we wanted a molecule that was small, well mannered and for which we had some reason to think might be structurally compatible with our first set of targets. Our choice was a small proteinase inhibitor, eglin c. This protein is exceptionally stable, has no disulfide bonds, is well characterized and binds very tightly to proteins similar to our first target which is stromelysin, a proteinase implicated in metastasis. Eglin c inhibits its normal targets, serine proteinases, by binding so tightly in the Michaelis complex, that the protein cannot be raised into the transition state. A ten amino acid loop in eglin c binds within the active site groove of the native serine proteinase targets.

High affinity binding requires a sequence (binding epitope) which is compatible with the target and a set of structural constraints on that sequence which prevent it from spending much time in non-productive conformations. The engineering task, which we have set ourselves, is to replace the wild-type binding epitope with one suitable for the target and then to construct a set of new constraints to move the binding epitope into the high affinity domain. Our initial target is stromelysin and hence an appropriate binding epitope is already known, that is, a substrate sequence preferred by the proteinase. Building an inhibitor then reduces to finding a suitable series of structural constraints that can be imposed by the eglin framework on the new binding epitope.

We are pursuing two sub-lines of investigation. One is to expand our information about eglin c as a suitable framework for protein engineering and the other is to start the protein engineering with what we already know.

BODY

Summary

During this fourth year of the project we have spent the bulk of our time on vector constructions, library constructions and working out methods to implement the new method we described last year called patterned library analysis. We have renegotiated our agreement with Parke-Davis for them to supply us with stromelysin since our previous contact, Dr. Ye, has left the company. We have constructed our first full scaffold display library. We have tested $CdCl_2$ inactivated stromelysin as a potential target for binding to alleviate potential cleavage of weak binders. An outline of our year's activities follows:

A. Stromelysin production

1. we have obtained 1.5 mg of purified stromelysin form Parke Davis

B. Library Construction and Screening

1. full framework peglin libraries
    a. we have modified the full scaffold peglin vector
        i. we removed two Ear I sites
        ii. we verified the modifications via sequencing
    b. we have constructed our first full scaffold display library (3×10^5 diversity)

C. Framework Characterization

1. we have worked out some of the conditions for implementing patterned library analysis
    a. we have redesigned the eglin activity (inhibition) assay
        i. improved dynamic range from 20-fold to 200-fold
        ii. the new assessment method gives reproducible results in various SDS and lysate concentrations in contrast to previous method
    b. we have shown that eglin variant activity is proportionate to variant stability
    c. we have shown that eglin denaturation is reversible in low levels of SDS
    d. we have shown that proteinase K can retain activity in SDS while losing its capacity to be inhibited by eglin c.

A. STROMELYSIN PRODUCTION

Last year we established an arrangement with Parke Davis wherein they provided us with active stromelysin for these studies. Our contact at Parke Davis, Dr. Ye, has now left the company. However, we have reestablished our arrangement and have received a second shipment of stromelysin (1.5 milligrams).

B. LIBRARY CONSTRUCTION AND SCREENING

We are using three types of binding epitope libraries for our analyses (Figure 1). The first is a traditional randomized peptide library. Our library has twelve randomized amino acids. As effective inhibitors peptides have several disadvantages. There is an upper limit on the binding affinity of an unstructured peptide of around 10^{-8} M. Most peptides bind with affinities, which are not useful under physiological conditions. Second, randomized peptides are very promiscuous in terms of locations to which they bind and hence many binders turn out not to be inhibitors. Third, there is a significant limit

to the selectivity of peptides to homologous proteins. The premise behind our approach is that epitopes displayed on structured frameworks and in particular on loops on structured frameworks can overcome many of these problems. Hence we desire to test binding epitope libraries on various frameworks to verify our assumptions.

B.1. Full Framework Libraries

The full framework protein we have chosen as a scaffold for displaying binding epitopes is eglin c. The expectation is that this small protein will provide the scaffolding for structural constraints giving rise to higher affinity binding than is attained with peptides. We also presume that by presenting the binding epitopes on a loop that they will more likely bind to pockets in the target protein than randomized peptides. Most pockets will contain the active site and hence the likelihood of binders being inhibitors is presumed to be good.

B.1.a. Peglin as the Scaffold Protein

In our first year we showed that eglin c did not, in fact, bind to its appropriate target in the phage display system. Our hypothesis was that the C-terminus of eglin (the site of fusion to the phage) was too close to the loop containing the target binding residues such that the phage would physically interfere with access to the binding epitope. Hence we designed a circularity-permuted version of eglin called peglin. Last year we showed that peglin does indeed bind to its native target as expected.

B.2.c. Library Construction Methodology

We have found that every time we change enzymes to accommodate the distribution of restriction sites for a library that it takes us a very long time to get the conditions adequate for making high diversity libraries. To reduce this optimization time we have shifted to a technology which allows us to use the same enzymes no matter where we need to make changes in the peglin gene. Hopefully, a single optimization will then apply to 'all' of our constructions.

The approach (Figure 2) makes use of a set of enzymes, which cut outside of their recognition sequence. Combining this with PCR technology, which allows one to amplify the entire plasmid, makes it possible to replace any portion of the peglin gene using the same set of enzymes. To use this approach we needed to remove two Ear I sites in our plasmid. These sites have been removed by site directed mutagenesis and the changes verified by sequencing.

B.3.c. Library Construction

We have constructed a library in peglin that contains eight randomized residues in the binding loop of eglin. This first full scaffold library has a diversity of 3×10^5 independent clones. This is a relatively low diversity and we are preparing to construct another library. This library is being tested against several inexpensive targets but the results are inconclusive at this time.

C. FRAMEWORK CHARACTERIZATION

C.1. Thermodynamic Characterization

We have continued our characterization of the protein, which serves as the framework for epitope display. This work has led to one new manuscript submission: "Effects of a Polyhistidine Terminal Extension on Eglin c Stability" by Waldner, Lahr, Edgell and Pielak. The results were reported last year in our annual report and the manuscript submitted this year.

C.2. Determinants of Structure in the Framework Protein Eglin c

One of the most powerful methods to characterize proteins is to utilize mutagenesis to test hypotheses. This approach has yielded considerable insight into the determinants of structure. However, despite these advances in our insights we are still unable to predict the consequences of most changes in a protein or to predict structure from primary sequence. During the course of this project it occurred to us that it might be possible to extend traditional mutagenic approaches to provide a quantitative assessment of hypotheses concerning protein structure. Our approach, which we call patterned library analysis, is conceptually quite straightforward. Consider a hypothesis about protein structure, e.g. the N-terminal 'cap' of an alpha-helix will consist of [T,S]$\pi\pi$G where π is any hydrophilic amino acid. It is now possible to construct a library of variants all of which are consistent with this hypothesis. If this hypothesis were in fact true then all of the variants in this particular library would have the target structure and hence would be fully active. On the other hand if the hypothesis were only partially true, e.g. perhaps only the thr works and not the ser in the N-terminal position, then not all of the variants would have the target structure and in this example only 50% would be active. That is, the fraction of the library, which is active, becomes a quantitative measure of the quality of the hypothesis. This simple concept gets complicated quite quickly as one considers the arbitrary nature of activity assays and the fact that proteins do not respond in an all or none way to amino acid replacements. However, we feel that the principle of the approach remains intact particularly when one applies this approach to not assess a single hypothesis but rather compares one to another to decide which is the better.

C.2.a. Inhibition Assay for Patterned Library Analysis

The patterned library analysis method we are developing depends on an efficient and reproducible assay for eglin inhibition and an assay system that can be used in SDS. Inhibition is measured by the reduction in capacity for a target proteinase, in our case proteinase K, to cleave a chromophoric substrate. In our initial assay we simply plotted the initial or maximum velocity of color production as a function of eglin concentration. Two problems presented themselves with this assay approach. First, the assay has a very limited dynamic range of about 20-fold. Secondly, the shape of the response curve changes in different conditions such as SDS concentration or when lysate is present. This means that if we want to measure how much eglin activity has been retained by eglin in say 0.01% SDS two different dilutions give values that differ by more or less than the dilution factor. We have been able to get around these two problems by using what is called a dose response assay. Vmaxs are measured for eight dilutions of each sample and the mid-point (EC_{50}) of the S-shaped curve is used as the metric for that variant. This approach has expanded the dynamic range of the assay so that it is now 200-fold.

We have used a scripting language (QuicKeys) to automate some of the complex calculations necessary to extract the EC_{50}s from the data.

C.2.b. Relationship between Protein Activity and Stability

We have measured protein stability for eleven eglin variants using CD-monitored denaturation in quanidine•HCl. A plot of the eglin equivalents measured at various SDS concentrations versus the $\Delta\Delta G$ of denaturation gives a straight line (Figure 3). This supports the presumption on our part that activity measurements can be used to generate a metric to compare one library of variants to another.

C.2.c. Denaturation of Eglin in Low Levels of SDS is Reversible

Thermodynamic properties of proteins can be determined in various ways. One of the most widely used is circular dichroism-monitored denaturation by temperature or increasing concentrations

of a denaturant such as guanidineHCl. The equations that are used to extract thermodynamic data from a denaturation curve require that the reaction be reversible. Since circular dichroism measurements require a large amount of material, that is, ~50 mg, we have been interested in using activity monitored denaturation. Hence we were very pleased to discover that denaturation in low levels of SDS was in fact reversible (Table 1).

TABLE I.
Relative activities (activity per molecule of eglin compared to purified eglin at 0% SDS) of eglin c after transferal to solutions at the indicated SDS concentration.

No SDS		0.08% SDS		Two-fold diluted
1.0	--->	0.16	--->	0.89
1.0	-----	----------	--->	1.04

Two samples of purified eglin were titered for activity (column one). One of the titered samples was brought up to 0.08% SDS, left for 30 minutes, and then titered in 0.08% SDS containing solutions. The relative activity after correction for the volume change was 0.16 of what it was in the absence of SDS. That sample, was then diluted by two in SDS free buffer and titered in SDS free solutions. Its relative titer after correction for the two fold dilution was 0.89. The other sample, that had never seen SDS, was also diluted by two and titered in SDS free solutions. Its relative activity corrected for dilution was 1.04.

C.2.d.Inhibition of SDS Treated Proteinase K by Eglin and the Activity of the Enzyme are Separable Functions

While testing various protocols for assaying eglin variants for inhibition in various concentrations of SDS we discovered that capacity to be inhibited and activity were separable properties of proteinase K. Proteinase K remains fully active in its capacity to cleave a chromophoric peptide substate after 2 hours of exposure to 0.08% SDS. However, when eglin is added to 2 hour treated proteinase K it is only half as effective as when it is added to 15 minute treated proteinase K. We will explore this in more detail later. Currently our hypothesis is that an SDS shell slowly forms about the proteinase K molecule that repeals eglin but not the chromophoric substrate.

C.2.e. Difficulties with the Dose Response Assay for Eglin Inhibition

We have started to collect multiple analyses on a set of variants for which we have stability data. The idea is to use this data to determine levels of reproducibility and to examine the data production process for any problems prior to application to many variants. An examination of the curve fitting at the higher SDS levels (0.03 to 0.045% SDS) indicates that the data points are not following the dose response curve to which we are trying to fit the data. Something is messing up the data.

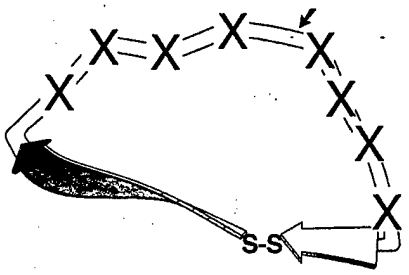
CONCLUSIONS

We need to show that the scaffold display approach can give rise to high affinity binders. This will require libraries with more diversity than we have attained so far. We will focus our next efforts on building higher diversity libraries.

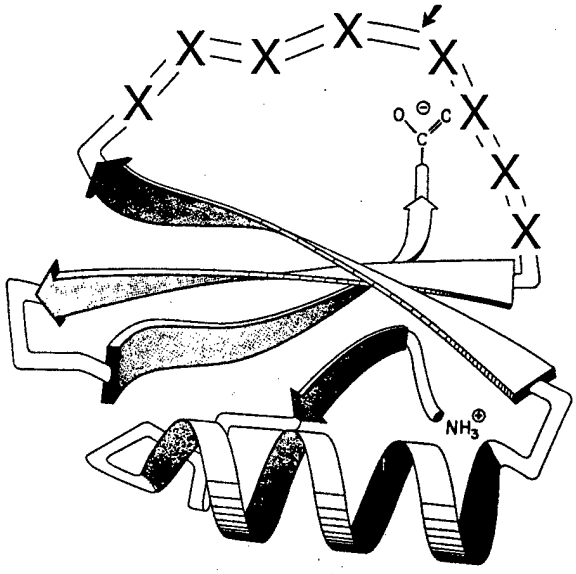
The patterned library analysis method is coming along nicely. There have been a surprising number of technical issues in producing a reproducible activity value for the variants. However, most of those problems appear to be resolved. In addition, the discovery that denaturation in low levels of SDS is reversible opens up the possibility that we can collect thermodynamic determinations on large numbers of eglin variants using activity monitored denaturation.

$(X)_{12}$

RANDOMIZED PEPTIDE



TEGLIN



EGLIN

Figure 1. Three Libraries Used For Screening.

The randomized peptide library has twelve N-terminal residues randomized and is fused to the phage gene pIII protein via a GGGG linker. The TEGLIN libraries are embedded in an 18 amino acid loop constrained via a disulfide bond. The non-randomized residues in teglin are derived from eglin. The loop sequences are tethered to the pIII protein via a QGGGG linker. The libraries based on EGLIN have the epitope sequences embedded in the same loop but now constrained by the entire framework and framework-loop interactions.

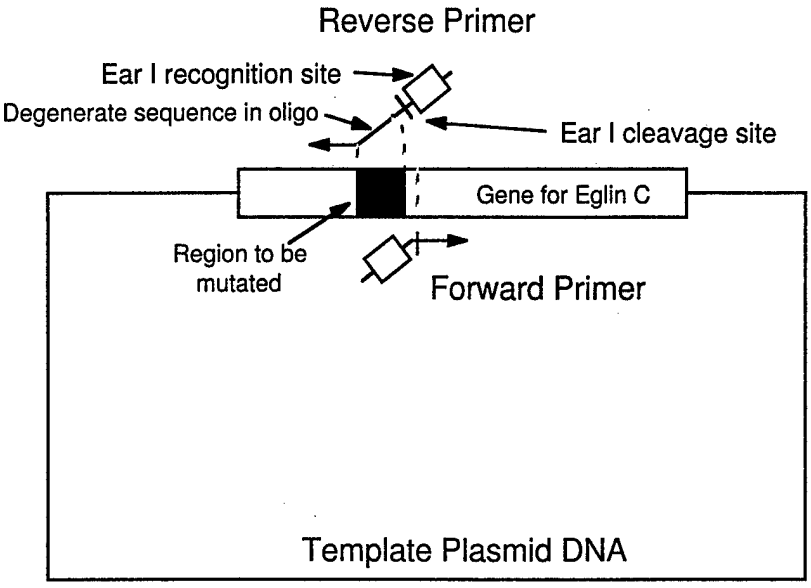


Figure 2. PCR Strategy for Library Production.

In this PCR based approach a reverse primer is synthesized that contains a degenerate region spanning the region in the gene to be mutated. That region does not hybridize to the template DNA (non-hybridizing regions of the primers are shown as lines that are not parallel to the template. The reverse primer also contains a short stretch of sequence that is homologous to the template and contains the sequence that will be cleaved by the Ear I enzyme. Ear I cleaves 3 nucleotides downstream of the Ear I recognition sequence which is contained in the reverse primer and hence will appear in the amplified product even though it is not present in the template. Enzymes like Ear I can in this fashion be used to cleave a template DNA and leave a 'sticky' end in any defined position of a template regardless of sequence. The forward primer is designed to have an Ear I sequence spaced in the primer oligonucleotide such that it will cleave the amplified product and leave an end that is complimentary to the end produced in the reverse primer region. Long PCR protocols allow one to amplify the large plasmids such as ours which is about 5000 bp in length.

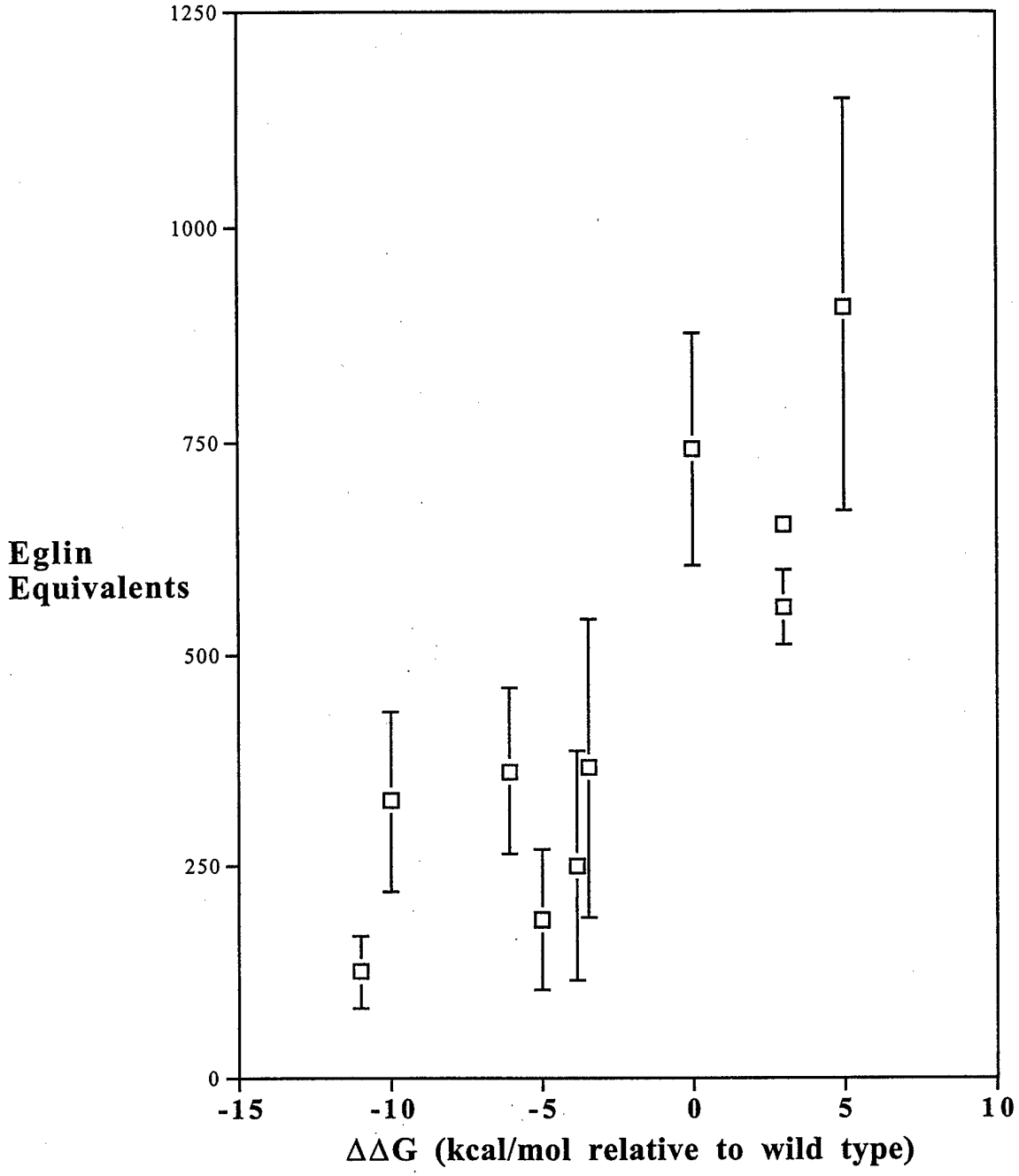


Figure 3. Variant Activity versus Stability. The activity of these variants was measured in 0.00% SDS using the dose response-based standard curve. The stability of these variants was determined using circular dichroism-monitored denaturation by temperature.

APPENDIX

In Press Manuscript
Accepted for Analytical Biochemistry

Effect of a Polyhistidine Terminal Extension on Eglin c Stability

by

Jennifer Waldner, Stephen J. Lahr, Marshall Hall Edgell, and Gary J. Pielak

Notes and Tips

Effect of a Polyhistidine Terminal Extension on Eglin c Stability

Jennifer C. Waldner[*], Steven J. Lahr[§], Marshall Hall Edgell[†], and Gary J. Pielak[*,1]

[*]Department of Chemistry, [§]Department of Biochemistry & Biophysics, and [†]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

[1]To whom correspondence should be addressed. FAX 966-3675; email gary_pielak@unc.edu.

Polyhistidine terminal extensions (his tags) are attached to recombinant proteins to facilitate purification because they bind resin-immobilized divalent cations, most commonly, Ni^{2+} (1). The effects of his tags on protein stability are idiosyncratic and rarely reported (2,3), so we set out to determine the effect of a six-residue N-terminal

tag on the thermal stability of the serine protease inhibitor eglin c (4). The tag does not effect eglin c stability.

Circular dichroism-detected thermal denaturations were performed between pH 1.5 and 3.3 for both proteins. The denaturation reaction is reversible and well fit by a two-state model (5). Values for T_m (the temperature at which the thermal transition is half complete) and ΔH_m (the vant Hoff denaturation enthalpy at T_m) are given in Table 1. The change in heat capacity upon denaturation, ΔC_p, was determined by varying the pH (5). Plots of T_m versus ΔH_m yield ΔC_p values for the wild-type protein and the tagged variant of 0.84 ± 0.07 and 1.13 ± 0.13 kcal mol^{-1} K^{-1}, respectively. The uncertainties were calculated from unweighted linear least squares fitting. ΔG_D (the free energy of denaturation) at each pH was calculated using a modified form of the integrated Gibbs-Helmholtz equation (6):

$$\Delta G_{D,T} = \Delta H_m\left(1-\frac{T}{T_m}\right) - \Delta C_p\left[\left(T_m - T\right) + T\ln\left(\frac{T}{T_m}\right)\right]$$

The uncertainty in ΔG_D was estimated by applying propagation of error analysis to the equation (5).

As shown in Figure 1, the tag does not affect eglin c stability. ΔG_D values at each pH are within the experimental uncertainty for each protein. A similar result is observed for the eglin c homologue, chymotrypsin inhibitor II (CI2), which possesses a natural 19-residue N-terminal tail (7,8). Deletion of this tail has a negligible effect on stability (9). Presumably, the tails do not affect stability because they are unstructured in both the native and denatured states.

In summary, the his tag does not affect the thermal stability of eglin c. This finding will facilitate our studies of eglin c variants obtained using a high throughput activity screen.

Materials and Methods

Expression and Purification. The eglin c gene was inserted into the pET17b vector (Novagen) and expressed using the *Escherichia coli* strain BL21(DE3)pLysS (10). After lysis, cell extracts were brought to pH 3.0 and insoluble proteins removed by centrifugation. Eglin c was purified from the supernatant by using a Sephadex G-75 gel filtration column equilibrated in 50-mM glycine HCl, pH 3.0.

Construction and Purification of tagged eglin c. The eglin c gene was removed from pET17b and inserted into the N-terminal His tag-containing vector, pET28a (Novagen).

Transformants were induced with 1 mM isopropyl β-D-thiogalactopyranoside. Purification was completed using Novagen's pET His-Tag system.

CD-detected thermal denaturation. Data were acquired with an Aviv model 62DS spectropolarimeter equipped with a five-position sample chamber. Experiments were performed in 50 mM glycine-HCl buffer using protein concentrations of 60 μM to 70 μM. The ellipticity at 227 nm was followed from 5 °C to 90 °C at 1 °C intervals, with ≈6.0 min between temperatures. Reversibility was checked by returning the samples to the initial temperature and repeating the experiment (5).

Acknowledgements. This work was supported by the US Army Medical Research and Material Command under DAMD17-94-J-4270 and by N.I.H. grant GM42501. JCW was partially supported by a G.A.A.N.N. Fellowship from the US Department of Education. We thank A. Broadwater and L. Hensley for technical assistance and the Pielak and Edgell groups for helpful discussions.

Figure Legend

Figure 1: ΔG_D-versus-pH plot for wild type(O) and his tagged (□) eglin c at 25.0 °C. The error bars were obtained as described in the text. The curves have no theoretical significance.

TABLE 1

Thermodynamic Parameters for Denaturation of Eglin c and His-tagged Eglin c

Protein	pH	T_m ±1.6[a] (°C)	ΔH_m ±6.3[a] (kcal/mol)
Wild-type eglin c	1.5[b]	45.1	45.3
	2.0[b]	47.3	43.8
	2.5[c]	54.6	51.2
	3.0[e]	62.2	57.4
	3.3[c]	69.0	64.3
His-tagged eglin c	1.5[c]	45.9	40.8
	2.0[c]	48.4	45.8
	2.5[c]	56.4	56.2
	3.0[d]	63.4	63.0
	3.3[c]	69.0	69.2

[a] Uncertainties are the standard deviation of the mean from four repetitions at pH 3.0 and are representative of the uncertainties at other pH values. [b] The average values from two, [c]three, [d]four, or [e]five trials.

REFERENCES

1. Hengen, P. N. (1998) *Trends in Biochemical Sciences* **20**, 286

2. Deutscher, S. L., Crider, M. E., Ringbauer, J. A., Komissarov, A. A., and Quinn, T. P. (1996) *Arch. Biochem. Biophys.* **333**, 207-213.

3. Ledent, P., Duez, C., Vanhove, M., Lejeune, A., Fonze, E., Charlier, P., Rhazi-Filali, F., Thamm, I., Guillaume, G., Samyn, B., Devreese, B., Van Beeumen, J., Lamotte-Brasseur, J., and Frere, J. M. (1997) *FEBS Lett.* **413**, 194-196.

4. Seemüller, U., Meier, M., Ohlsson, K., Müller, H. P., and Fritz, H. (1977) *Hoppe Z. Physiol. Chem.* **358**, 1105-1107.

5. Cohen, D. S. and Pielak, G. J. (1994) *Protein Sci.* **3**, 1253-1260.

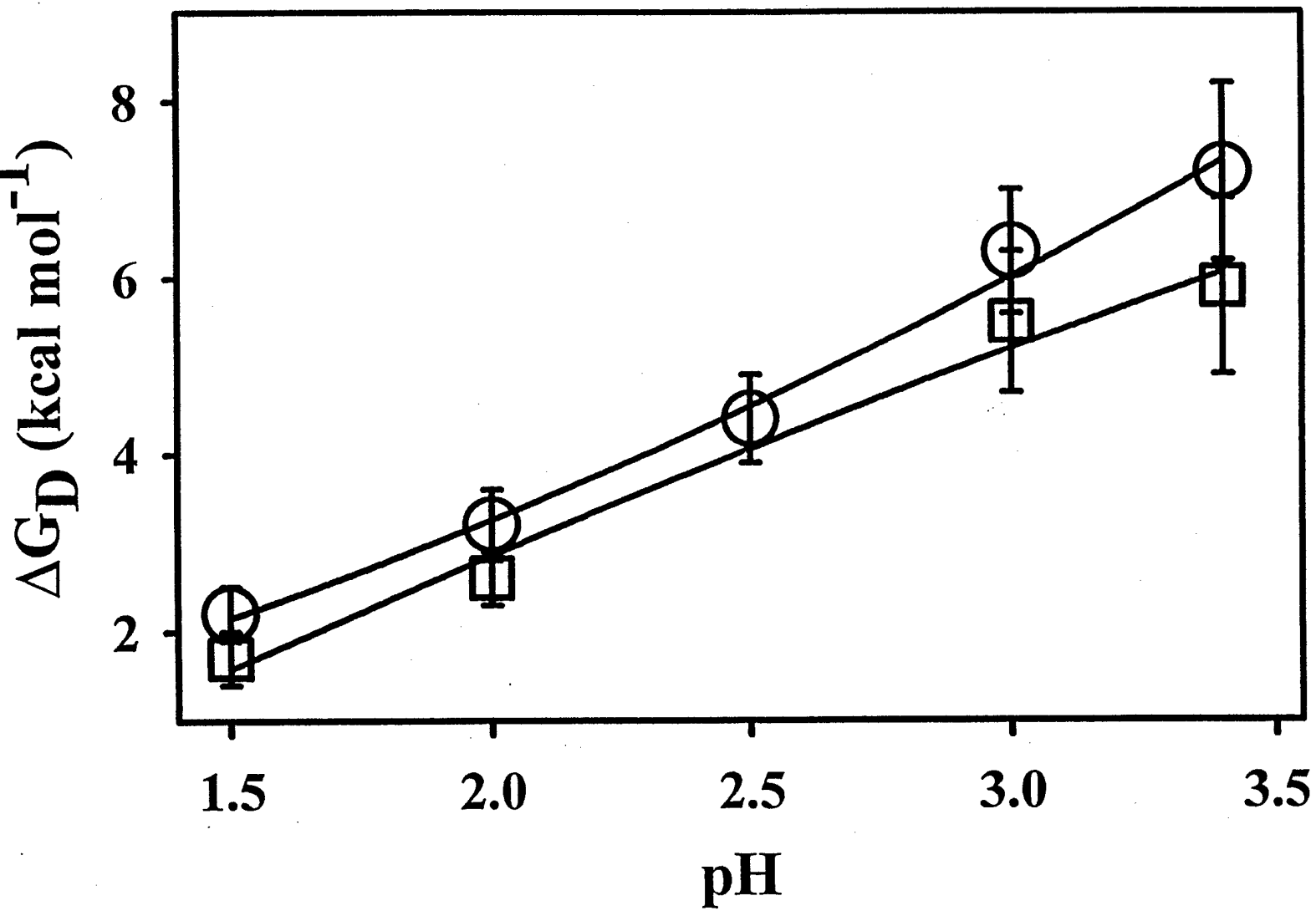
6. Elwell, M. L. and Schellman, J. A. (1977) *Biochim. Biophys. Acta* **494**, 367-383.

7. Kjaer, M., Ludvigsen, S. J., Sorenson, O. W., Denys, L. A., Kindtler, J., and Poulsen, F. M. (1987) *Carlsberg Res. Commun.* **52**, 327-354.

8. Kjaer, M. and Poulsen, F. M. (1987) *Carlsberg Res. Commun.* **52**, 355-362.

9. Jackson, S. E., Moracci, M., elMasry, N., Johnson, C. M., and Fersht, A. (1993) *Biochemistry* **32**, 11259-11269.

10. Grodberg, J. and Dunn, J. J. (1988) *J. Bacteriol.* **170**, 1245-1253.


8
6
4
2
ΔG_D (kcal mol^{-1})
1.5
2.0
2.5
3.0
3.5
pH